Altered status of CD4\(^+\)CD25\(^+\) regulatory T cells in patients with acute coronary syndromes

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Aims Considerable evidence supports the role of innate and adaptive immunity in the progression and destabilization of the atheromatous plaque. Naturally occurring CD4\(^+\)CD25\(^+\) regulatory T cells (Tregs) are a subpopulation of lymphocytes that are capable of suppressing the progression of experimental autoimmune disorders. We have hypothesized that peripheral numbers and function of Tregs would be deranged in patients with acute coronary syndromes (ACS).

Methods and results Peripheral numbers of Tregs were evaluated by FACS employing labelled antibodies to CD4 and CD25. Functional suppressive properties of Tregs were assayed by establishing a triple-cell culture in which purified Tregs were incubated with irradiated antigen-presenting cells and anti-CD3-activated responder T cells. Proliferation in the presence or absence of oxidized LDL (oxLDL) was evaluated by thymidine incorporation. mRNA and protein content of foxp3, a master transcriptional regulator of Tregs, were determined for all subjects. Patients with ACS exhibited significantly reduced numbers of peripheral Tregs as compared with patients with stable angina and normal coronary artery subjects. Moreover, oxLDL induced a more profound reduction in Treg numbers in patients with ACS. Tregs in ACS patients were significantly compromised as their ability to suppress responder CD4\(^+\)CD25\(^+\) T-cell proliferation was attenuated. mRNA and protein content of foxp3 were significantly reduced in purified Tregs obtained from patients with ACS.

Conclusion In patients with ACS, naturally occurring CD4\(^+\)CD25\(^+\) Treg numbers are reduced and their functional properties compromised. These findings may aid in understanding the mechanisms leading to culprit plaque associated T-cell activation in patients with ACS.

KEYWORDS Atherosclerosis; T cells; Immune response; Acute coronary syndrome; Foxp3

Introduction Atherosclerosis is a multifactorial disorder in which immune mechanisms appear to play a dominant role. This finding was evident by observing T cells and antibodies at different stages of advancing atheromatous plaques.\(^1\)\(^-\)\(^3\) One of the byproducts of this relatively recent idea is that autoimmune-like processes may be involved in shaping the size and composition of the atherosclerotic lesion. Indeed, experimental data from animals indicate that immunization against antigens such as heat shock protein 60/65 and beta2 glycoprotein I result in accelerated atherosclerosis.\(^4\)\(^-\)\(^6\) Moreover, transfer of lymphocytes, reactive to these respective antigens or with no antigen specificity, promotes early atherosclerosis in mice.\(^7\)\(^-\)\(^9\)

In humans, evidence for autoimmune involvement is found in studies in which atherosclerotic subjects appear to exhibit higher titers of autoantibodies to HSP60/65,\(^10\) and these antibodies could also predict prognosis.\(^11\) A possible causative role for autoreactive T cells is suggested by studies, showing that considerable number of T cells are present in human and murine plaques,\(^12\)\(^-\)\(^13\) and some are reactive with oxidized LDL (oxLDL) and exhibit a T helper 1 phenotype upon antigen-specific stimulation.\(^14\)

Experimental, but indirect, data suggests that induction of Tregs may attenuate the progression of atherosclerosis. This is inferred by studies suggesting that oral tolerance with potentially proatherogenic antigens reduces the extent of atherosclerosis,\(^15\)\(^-\)\(^17\) possibly through T helper cell phenotype shift and from a set of studies showing that cytokine products of Tregs are anti-atherogenic.\(^1\)\(^-\)\(^3\)\(^,\)\(^18\)

It is now recognizable that most plaques that cause acute coronary syndromes (ACS) exhibit angiographic obstruction of less than 70% (reviewed by Naghavi et al.\(^19\)\(^,\)\(^20\) Approximately 60% of ACS-related atheromas are caused by rupture of plaques with a large, thrombogenic core of lipid and necrotic debris (including foci of macrophages, T cells, old haemorrhage, angiogenesis, and calcium). The factors that govern the transition of the plaque from a stable to a rupture-prone lesion are not sufficiently understood. However, mounting evidence exists to support the role of immune system dysregulation, either due to extrinsic or intrinsic factors, in the alteration of plaque phenotype.
In recent years, considerable evidence has implicated naturally occurring CD4^+ CD25^+ Tregs in the control of autoimmunity and adoptive transfer of purified Tregs-affected protection from experimental autoimmune disorders. Tregs have also been demonstrated in peripheral blood of normal coronary arteries (NCA) subjects. Several recent studies have reported a decrease in the number of CD4^+ CD25^+ Tregs in peripheral blood of patients suffering from systemic lupus erythematosus, type-1 diabetes, rheumatoid arthritis (RA), and multiple sclerosis.

In the current study, we tested the hypothesis that the pool of naturally occurring CD4^+ CD25^+ Tregs is dysregulated in patients with ACS. The study was also aimed at investigating the comparative expression of foxp3, the transcriptional regulator of Tregs.

**Methods**

**Patients**

Institutional Ethics Committee approved the study and informed consent was obtained from all patients. Three groups of subjects were selected: group 1, ACS patients admitted to the intensive coronary care unit (n = 32); group 2, patients with stable anginapectoris and angiographically documented atherosclerosis (n = 28); and group 3, subjects with NCA on angiography (n = 28) (Table 1).

ACS was defined as chest pain accompanied by definite ischaemic electrocardiographic changes (ST-segment changes and/or T-wave inversions). Myocardial infarction was diagnosed if there was also elevation of troponin I (>$0.1$ U) or CKP MB ($>$ or definite ($>2$ mm) ST-segment elevations in at least two consecutive leads. Patients with stable angina were recruited from the outpatient clinic and a recent angiography exhibited coronary afflication of a similar extent to that found in ACS patients. Control subjects were selected on a basis of a recent angiography showing NCA.

Exclusion criteria were: age below 18 years or above 80 years, renal failure, serum creatinine above 2 mg/dL, known history of cancer or chronic-immune-mediated disorders, or current use of immunosuppressive agents including corticosteroids. A total of 32 ACS patients, 29 stable angina patients, and 28 subjects with NCA were recruited, from which 32, 28, and 28, respectively, were selected based on the exclusion and inclusion criteria. Patients with ACS and stable angina had similar extent of coronary atherosclerosis. No differences were evident between the three groups with ACS and stable angina had similar extent of coronary athero-

**Table 1** Characteristics of the study patients

<table>
<thead>
<tr>
<th></th>
<th>ACS (n = 32)</th>
<th>Stable angina (n = 28)</th>
<th>NCA subjects (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>16 (50)</td>
<td>16 (57)</td>
<td>15 (54)</td>
</tr>
<tr>
<td>CAD extent (n x vessels)</td>
<td>2 ± 0.7</td>
<td>1.8 ± 0.9</td>
<td>0</td>
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<tr>
<td>Risk factors</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>16 (50)</td>
<td>12 (43)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>7 (22)</td>
<td>8 (29)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>8 (25)</td>
<td>8 (29)</td>
<td>2 (7)</td>
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<tr>
<td>Past smoker, n (%)</td>
<td>2 (6)</td>
<td>2 (7)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Hyperlipidaemia, n (%)</td>
<td>18 (56)</td>
<td>17 (61)</td>
<td>5 (18)</td>
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<tr>
<td>Medications</td>
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<td></td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>10 (31)</td>
<td>12 (43)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>ACEI, n (%)</td>
<td>16 (50)</td>
<td>15 (53)</td>
<td>3 (11)</td>
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<tr>
<td>Aspirin, n (%)</td>
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</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>0 (0)</td>
<td>3 (11)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Data mean ± SD.

CABG coronary artery bypass grafting; PTCA, percutaneous transluminal coronary angioplasty; CVA cerebrovascular accident; ACEI, angiotensin-converting enzyme inhibitors; ARB angiotensin receptor blocker.

**Functional suppression assays**

Costar 96-well plates (Corning, NY, USA) were incubated with $1 \mu g$ mL anti-CD3 monoclonal antibody (UCHT1 from R&D systems) overnight at 4 °C, and washed. Then, CD4^+ CD25~+~ (responders) and CD4^+ CD25~+~ (Tregs) T cells (10^5^ cells/well) were cultured in RPMI medium supplemented with 10% foetal calf serum in different responder/suppressor ratios (1:1, 1:1/2, 1:1/4, and 1:1/8). All cells were cultured in a final volume of 200 μL in the presence of 10^5^ T cell-depleted (peripheral mononuclear cells) and irradiated antigen-presenting cells/well. After 72 h, 3H-thymidine (1 μCi/ well) was added for 16 h before proliferation was assayed by scintillation counting (β counter). Percent inhibition of proliferation was determined as follows: 1 – (median [H]-thymidine uptake of 1:1 CD4^+ CD25~+~:CD4^+ CD25~+~ coculture/median [3H]-thymidine uptake of CD4^+ CD25~+~ cells).

The coculture/proliferation assay for assessment of the functional suppressive properties of Tregs was repeated in the presence or absence of oxoLDL (1 mg/mL).

**Foxp3 and CTLA-4 expression determined by reverse transcription–polymerase chain reaction**

RNA was extracted from 10^5^ T cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The amount and purity of the obtained RNA was determined by measurements of optical density (OD) at 260 and 280 nm. Reverse transcription–polymerase chain reaction (RT-PCR) was performed according
to the protocol of Reverse-iT First Strand synthesis kit (ABgene, UK). The integrity of the RNA, the efficiency of RT reaction, and the quality of cDNA subjected to the RT-PCR was controlled by amplification of transcript of GlycerAldehyde-3-Phosphate Dehydrogenase (GAPDH). GAPDH was analysed using the following primers: GAPDH forward 5'-ACCACAGTCCATGCCCAC-3' and GAPDH reverse 5'-TCACACCACACCTGGTGCTGA-3'.

PCR was carried out with ReadyMix PCR mastermix (ABgene, UK) on programmable thermal controller (PTC) device (MJ Research inc) at gene specific conditions. Primer sequences for foxp3 were: foxp3 forward 5'-CACTTGAGACACATTGC-3' and foxp3 reverse: 5'-CTCTTTCTTTGAAACCCA-3', and for cytotoxic T-lymphocyte antigen-4 (CTLA-4) forward 5'-GCTATGGGCCGAGTAAGTG-3' and CTLA-4 reverse 5'-CTGCTTCTGAAAGGAATG-3'.

The PCR products were subjected to electrophoresis on 1.5% agarose gel stained with ethidium bromide. The OD of the amplified PCR product was measured by densitometry. Semi-quantitative analyses presented comparison of OD of FoxP3 and CTLA-4 PCR products normalized to OD of co-amplified GAPDH-PCR product.

**Western blot analysis of foxp3 protein content in Tregs from patients with ACS, stable angina, and NCA subjects**

Purified Tregs from all patients were lysed, and protein concentration in lysates was determined using BCA protein kit (Pierce USA). Cell lysates were resolved on 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Western blot was performed using a rat serum anti-foxP3 (eBioscience, USA) at a dilution of 1:1000, and a secondary antibody-peroxidase-conjugated AffiniPure donkey anti rat IgG (H + L) (Jackson Laboratories) for detection with chemiluminescent substrate (Santa-Cruz, USA). Comparative analysis was performed by quantitative densitometry.

**Statistical analysis**

Comparison between the three patient groups was carried out employing the one-way ANOVA test with the Newman Keuls post test and the dunnett adjustment test. Statistical significance was set at a double-sided P-value of <0.05. Results are reported as mean ± SD unless otherwise specified.

Sample size calculations indicated that groups comprising 22 patients were sufficient to detect a difference of 30% in Treg numbers between groups with 80% power at the 5% significance level.

**Results**

**OxLDL influences Tregs number and function**

OxLDL is considered an instrumental factor that promotes atherosclerosis initiation, progression, and possibly, plaque destabilization.1-3 We studied the effects of different concentrations of oxLDL on relative CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ cell numbers after in vitro incubation (Figure 1A). Tregs were significantly more sensitive to oxLDL as their relative number was reduced by 40 ± 8% in comparison to a negligible effect on CD4⁺CD25⁻ cells (15 ± 5%). When the assay was repeated in the presence or absence of the caspase inhibitors DEVD-CMK (Calbiochem, La Jolla, CA, USA) or NAC (Sigma, St Louis, MO, USA), we have found that the effect of oxLDL on reduction of Tregs was attenuated producing a 17 ± 6% or 20 ± 6% reduction in regulatory and effector T cells, respectively, suggesting that apoptosis could have been partially responsible for the effect.

We then investigated whether Tregs from NCA subjects are compromised in their suppressive properties when exposed to oxLDL. Indeed, incubation of Tregs with 1 mcg/mL oxLDL, resulted in a significant attenuation of their ability to suppress CD4⁺CD25⁺ proliferation at all Treg-T-responder ratios (Figure 1B).
Circulating CD4+CD25+ T cell numbers are reduced in patients with ACS

Freshly drawn human ficoll-eluted blood mononuclear cells were stained with different combination of anti-CD4-FITC and anti-CD25-PE (Figure 2A and B). The cells were gated on lymphocytes via their forward- and side-scatter features. We have found that in patients with ACS, the number of CD4+CD25+ Tregs was significantly reduced (1.9 ± 1.1%) as compared with patients with stable angina with a similar extent of coronary affliction (3.2 ± 1.0%, P < 0.001) and individuals with NCA (4.0 ± 1.3%, P < 0.001). Treg numbers were not significantly different in patients with stable atherosclerotic disease and subjects with NCA.

CD4+CD25+ Tregs are functionally compromised in patients with ACS

As the functional suppressive properties of Tregs may be as important as their numbers, we isolated highly pure CD4+CD25+ regulatory and CD4+CD25- responder cell populations by magnetic bead sorting (>95% purity). CD4+CD25+ responder cells from patients with ACS, stable angina, and individuals with NCA exhibited similar proliferation, evident by thymidine incorporation to plate-bound anti-CD3 antibodies (Figure 3). CD4+CD25- T cells isolated from all groups were anergic to stimulation at all doses by plate-bound anti-CD3 and did not differ (1889 ± 378 cpm-NCA, 2394 ± 306 cpm-stable angina and 1703 ± 215 cpm in ACS patients).

Quantitative analysis of the regulatory function of CD4+CD25+ Tregs was performed by co-culturing them with autologous T-responder cells (10⁶ cells/well) at different ratios (responder/suppressor ratios: 1:1, 1:1/2, 1:1/4, and 1:1/8). The assay was repeated for all subjects. Similar to other investigators, we found that in NCA individuals, CD4+CD25- T cells suppressed responder T-cell proliferation at a 1:1 ratio and the effect was diluted by reducing the relative numbers of Tregs.

Tregs isolated from the circulation of patients with ACS exhibited hampered inhibition of responder CD4+CD25+ T cell proliferation when compared with Tregs from patients with stable angina pectoris or NCA individuals (Figure 3). For example, at a 1:8 Tregs-T-responder ratio, mean inhibition of proliferation of Tregs from ACS patients was 11.8 ± 7.3% as compared with 29.6 ± 9.2% achieved for stable angina pectoris and 58 ± 2.6% for NCA individuals (Figure 3). Interestingly, the functional suppressive properties were significantly compromised in patients with stable atherosclerotic disease as compared with NCA subjects (Figure 3).

OxLDL induces a differential effect on Treg numbers in patients with ACS, stable angina, and NCA subjects

We then investigated the hypothesis that differential sensitivity to oxLDL-mediated Treg depletion exists between patients with ACS, stable angina, and NCA individuals. We have found that CD4+CD25+ T cells from patients with ACS were significantly more sensitive to oxLDL-mediated depletion (a mean of 31% depletion) as compared with

Foop3 protein content in Tregs from patients with ACS

Western blot analysis of foxp3 protein content in Tregs from patients with ACS (Figure 5A). We have found that foxp3 expression was significantly reduced in purified Tregs from patients with ACS as compared with NCA subjects (a mean of 68% reduction, P = 0.04) and to patients with stable angina (a mean of 56% decrease, P = 0.038). We also assayed expression of CTLA-4, an additional potential phenotypic marker of CD4+CD25+ (Figure 5A). We have found that the expression of CTLA-4 was also significantly reduced in Tregs from ACS patients as compared with both NCA subjects and patients with stable angina (a 64% reduction and a 39% reduction, respectively, P = 0.032 and P = 0.041).
Discussion

In the current study, we tested the hypothesis that the number and function of Tregs in patients with ACS and stable atherosclerotic coronary disease is altered. Several lines of evidence support a role for regulatory cells in atheroprotection. (i) Induction of experimental oral tolerance in mice is associated with attenuation of atherosclerotic lesions, whereas transfer of antigen-specific lymphocytes leads to enhanced atherogenesis. (ii) In humans with unstable angina, cytokines that represent ‘signatures’ of Tregs are reduced, and these same cytokines are atheroprotective in experimental murine models.

Our findings suggest, for the first time, that peripheral, naturally occurring Tregs in patients with ACS are significantly reduced. In order to determine whether atherosclerosis per se, or events culminating in plaque destabilization are responsible for the effect observed on Tregs, we also studied patients with stable angina that had a similar extent of coronary atherosclerosis. Obviously, these results cannot establish a causal role for Treg-pool depletion in events that lead to plaque rupture and consequent ACS, yet they may offer new research insights.

Figure 3 Comparative analysis of peripheral T-responder and Treg proliferative capacity in patients with ACS. Purified responder T cells (CD4⁺CD25⁻) and purified Tregs activated by plate-bound anti-CD3 were assayed comparatively for their proliferative capacity between the three groups. Assay was performed for each of the patients in all three groups. Results of mean proliferation at each of the ratios indicated in Methods section are demonstrated for patients with ACS, stable angina pectoris, and NCA.

Figure 4 OxLDL induces a more profound reduction of Tregs from ACS patients. Coculture assay was performed, as described in the Methods section, also in the presence of 1 mcg/mL oxLDL. Figure shows the average reduction of Treg numbers after a 3-day coculture with oxLDL.
cell-contact inhibition, whereas antigen-specific Tregs act by cytokine secretion.\textsuperscript{21,39} We have employed the currently accepted methods for assessment and Treg function in the three patient groups. Tregs from patients with ACS were clearly compromised with regard to their suppressive function on responder T-cells, when compared with Tregs from NCA controls or patients with stable angina. We ruled out the possibility of a more robust proliferative activity of CD4\textsuperscript{+}CD25\textsuperscript{–} in patients with unstable angina by evaluating their thymidine uptake in the three patient groups and found essentially similar values. It is noteworthy, that on a 1:1 and 1:2 T-effector:Treg ratios, a compromise in the suppressive effect of Treg was not evident in ACS as compared with stable angina patients. However, since the numbers of Tregs in vivo are by far lower than effector T cells, the relevant concentrations are the 1:8 ratios and lower, in which clear, hampered, suppressive properties was found in Tregs from ACS patients.

Interestingly, whereas the number of peripheral Tregs from patients with stable coronary atherosclerosis was similar to that of NCA subjects, suppressive function of their CD4\textsuperscript{+}CD25\textsuperscript{–} Tregs was hampered when studied comparatively. The relevance of the proliferative effect of responder T cells to the progression of atherosclerosis is questionable. However, it has been shown that transfer of antigen-specific\textsuperscript{8,9} and non-specific\textsuperscript{7} (most of which are CD4\textsuperscript{+}CD25\textsuperscript{–}) lymphocytes increases plaque size in mice, whereas CD4 cell ablation\textsuperscript{41} and cross-breeding of atherosclerotic with RAG\textsuperscript{−/−} mice\textsuperscript{42} results in attenuation of atherosclerosis. It can thus be hypothesized that dysregulation of Treg-pool may be causally associated with the progression of atherosclerosis and plaque destabilization.

We aimed at investigating the potential role of oxLDL on the number and functional properties of Tregs. We found that oxLDL induced a more selective reduction of Tregs as compared with CD4\textsuperscript{+}CD25\textsuperscript{–} cells suggesting that the former population is more susceptible to the influence of oxLDL. Interestingly, oxLDL hampered the suppressive effect of purified Tregs on responder T-cell proliferation. We found that Tregs from patients with ACS were significantly more sensitive to oxLDL-mediated compromise of their numbers as compared with Tregs from NCA subjects and those with stable atherosclerosis. If a cause and effect relationship is to be further pursued, then it could be argued that differential sensitivity to oxLDL results in Treg depletion that promotes plaque inflammation and destabilization in ACS.

Foxp3 has received considerable interest in recent years being the master transcriptional regulator of Treg homeostasis.\textsuperscript{41} We thus hypothesized that it would be downregulated in purified Tregs from patients with ACS. Indeed, we have found by RT–PCR and western blot that purified Tregs from

\textbf{Figure 5} Expression of foxp3 and CTLA-4 mRNAs in Tregs of patients with ACS. Total RNA was isolated from purified Tregs of patients with ACS, stable angina pectoris, and NCA, reverse transcribed (as described in Methods) and assayed for foxp3 and CTLA-4 mRNAs expression levels. Results represent mean ± SEM of values obtained from densitometric analysis with GAPDH employed as the reference housekeeping gene (A). Purified Tregs from each group were assayed for foxp3 protein content by western blot. A representative blot from three arbitrary subjects (serial numbers shown) from each group is shown (B) and cumulative results (mean ± SD) of the densitometric analyses (C). *P < 0.05.
ACS patients exhibited a significantly reduced expression of foxp3. This finding was accompanied by the observation that expression of mRNA for CTLA-4, an additional marker that is thought to mirror Tregs,\(^{43,44}\) is also downregulated in the compromised Tregs from patients with ACS. Collectively, these findings may suggest that in patients with ACS, oxLDL may compromise the number and function of Tregs, by downregulating foxp3. Loss of suppression of Treg on potentially proatherogenic populations of CD4^+CD25^+ Tregs could consequently result in plaque destabilization and rupture.

In conclusion, we observed a significantly hampered Treg population in patients with ACS that exhibit a reduced expression of foxp3. These findings may suggest a potential role for Tregs in the progression and stability of the atheroma.

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**Conflict of interest:** None of the co-authors have conflicts of interest to disclose.

**References**


